Discussion
Autoimmune diseases are characterized by the presence of circulating autoantibodies which are not necessarily pathogenic but represent markers of immune mediated injury (Rosa, R and Bona, C, 1991). Autoimmune diseases result due to humoral and/or cellular immunological reactions against the self components of an individual. The triggering mechanisms of autoimmune reaction have not yet been completely understood. Shoenfeld and Isenberg (1989) described the wide spectrum of autoimmune diseases as mosaic of autoimmunity with many factors leading to various diseases.

Dilated cardiomyopathy (DCM) is a chronic heart muscle disease of unknown etiology characterized by dilatation and contractile dysfunction of left and/or the right ventricle. In man, myocarditis is known to be the precursor of DCM in many cases, although clinical and histological diagnosis remain problematic. Since in DCM, the only affected organ is heart, it fits into the criteria of organ specific autoimmune disease. Autoimmune features in patients with DCM include familial aggregation (Keeling et al., 1995), a weak association with HLA DR4 (Carlquist et al., 1991) and immunoglobulin genes (Martinetti et al., 1992), abnormal expression of HLA class II on cardiac endothelium (Caforio et al., 1990), and increased levels of circulating cytokines (Limas et al., 1995).

Several groups have found cardiac antibodies to various antigens in DCM (Caforio et al., 1992; Schultheiss et al., 1990; Limas et al., 1995). Multiplicity of parallel autoimmune reactions directed towards a single organ may be due to spread sensitization (Bach, 1995). Among the various
cardiac antigens, 150 kD C protein has been found to be a potent antigen causing autoimmune myocarditis in experimental animals. C protein plays an important role in contractile activity. It is one of the major constituent proteins which are essential to compete with natural antigens at MHC - antigen bindings site (Demotz et al., 1990; Harding and Unanue, 1990). Moreover C protein is an intracellular member of immunoglobulin superfamily (Einheber and Fischman, 1990). Due to identical molecular weights IgG, and C protein gave a similar pattern on 7.5% SDS - PAGE. Urea denaturation studies also showed more or less similar patterns in Urea - SDS - PAGE. When SDS - PAGE was performed with a urea gradient (1-10 M urea) as well as acrylamide gradient (5-20%), both IgG and C protein were denatured to give two prominent bands each. This further gives an insight into this theory. The HLA binding motifs are located in the variable domain and not the constant IgG like domain (Rudensky et al., 1992; Baum et al., 1993).

The thermal behaviour of 150 kD C - protein and human IgG as control displaying Tm values of unfolding at around 79.5°C is typical of a highly stable dimeric protein molecule. The highly stable conformational state of 150 kD C - protein was evident from the observation that only 4.4% had undergone thermal unfolding till 75°C. Thus our observation reveals for the tremendous structural stability of the 150 kD C - protein auto antigen.

Additional evidence for the structural changes in native 150 kD C-protein as a result of thermal energy supplementation was revealed by analyzing the data and computation of thermodynamic parameters (Table 1 & 2). The thermodynamical data speculate the tremendous structural stability
exhibited by 150 kD C - protein. Disruption of the hydrophobic and ionic interaction as well as disulfide bonds in the 150 kD C - protein which ultimately resulted in unfolding was inferred from the shift in the scales of the AG₃ values. The negative AG₃ values above 79°C suggests the transitions of completely folded 150 kD C - protein to the unfolded state. Furthermore, in comparison to the already established thermodynamic behaviour of native DNA, our results are indicative for the 150 kD C - protein to be topologically less constrained than nucleic acids. This comparative analysis has been pointed out due to the important factor of DNA acting as auto antigen in SLE whereas 150 kD C-protein acting as autoantigen in DCM / myocarditis and that as per our observations, the 150 kD C - protein which acts as autoantigen in myocarditis was found to be equally reactive with naturally occuring SLE autoantibodies.

Photochemical reaction have been found to produce DNA - protein crosslinks in biological systems. Proteins and nucleic acids are colourless macromolecules which absorb in the UV range of spectrum. These two biomolecules coexist in vivo and their interaction results in biological damage in organisms. The crosslinking between DNA and protein has been found to be covalent in nature. (Oleinick et al., 1986). In this study, we have formed photoadducts between native DNA and C - protein in UV light (254 nm). Time course kinetics revealed that the formation of photoadduct increased with increase in duration of exposure to UV irradiation. This was further reiterated by the amount of total protein bound to DNA. The protein content increased remarkably with the increase in period of exposure. The amount of lysine residues present in C-protein were estimated to assess their
binding to native DNA. Time course kinetic study indicates that the formation of photoadduct obeys apparent first order reaction.

Oxygen free radicals (OFR's) occupy a prime position in the world of free radical biochemistry. The *prima donna* in this category are oxygen itself, superoxide, hydrogen peroxide, transition metal ions and hydroxyl radicals, where the first four interact in many ways to produce the last (Halliwell and Gutteridge, 1990). Exogenous sources like toxic foreign compounds and ionizing radiations are known to increase the production of free radicals. There are several sites for generation of reactive oxygen species (ROS) in cells, but the main source is the autooxidation of reduced components of mitochondrial transport chain (Haiku et al., 1993).

Reactive oxygen species produce DNA-protein crosslinks in biological systems. Here we have taken two entities, hydrogen peroxide ($H_2O_2$) and hydroxyl radical (OH) to produce crosslinks and photoadduct formation between native DNA and C-protein. Among the two, hydroxyl radical (OH) appeared to be a better agent to induce DNA-C-protein crosslinks. The formation of photoadduct in the above cases causes single strand breaks in DNA as revealed by nuclease S1 sensitivity assay. On the contrary, the control and treated samples of nDNA C-protein photoadduct showed the same pattern. It signifies that in the photoadduct, interaction may have taken place via lysine residues. The other aminoacids in the protein can also undergo photointeraction to form protein-protein photoadduct. Thus, crosslinks are again formed between nDNA C-protein and C-protein C-protein photoadduct which is responsible for such a pattern.
The recognition between nucleic acid and proteins is of great significance as it triggers most important steps of nucleic acid metabolism. This recognition involves electrostatic interactions between phosphate groups and positively charged groups in protein. Later more specific interactions between nucleic acid bases (or other structural elements) and amino acid side chains in proteins take place. A closer look reveals the possible recognition of secondary structure of nucleic acids by proteins, either way recognition is dependant of nucleotide sequence (Duguet, 1981).

A different chemistry arises when protein and DNA are irradiated together or separately. Since DNA and protein are in intimate contact with each other in vivo, it is suggested that photochemical interaction of DNA and protein would play a significant role in the inactivation of UV irradiated cells under certain conditions.

The photoadducts formed showed appreciable recognition with DCM, SLE and immune sera. The reactivity of DCM and immune sera was maximum with photoadduct formed by hydroxyl radical (OH) followed by the one formed only in the presence of UVB irradiation showed least reactivity as shown by band shift assay and completion ELISA studies. This indicates the effectiveness of hydroxyl radical (OH) to generate DNA protein crosslinks which produce pathological changes.

The sera of patients with dilated cardiomyopathy (DCM) showed a typical picture in the biochemical investigations. Quantitative determination of serum cholesterol gave normal values except for one patient. The determination of serum cholesterol is considered to be significant mostly
in coronary artery diseases, hyperlipoproteinemas, hypothyroidism, nephrosis, diabetes mellitus, and various liver diseases, and has very little role to play in dilated cardiomyopathy (DCM). Similarly patients with DCM rarely show an increase of serum glucose. Only two of the total fifteen cases showed a moderate increase in glucose levels. The moderate rise is associated with infectious diseases, intracranial diseases and haemorrhage. There was a significant increase in uric acid levels in serum. Serum or plasma uric acid determination has an important diagnostic values in gout and kidney failure. It is also increased in acute stages of infectious diseases, severe uremia, toxemia of pregnancy and leukemia. DCM is a end stage cardiac disorder in which there is active degeneration of cardiac tissue leading to heart failure. This chronic inflammatory process may be responsible for increase in uric acid levels in DCM patients.

A significant rise was observed in the C - reactive protein (CRP) levels in sera of DCM patients. A majority of patients gave a high titre on semi - quantitative determination of CRP in serum. CRP an acute phase reactant is considered to be the most sensitive marker in the acute phase and therefore, a universal early indicator for inflammatory, necrotic and neoplastic diseases. CRP is a non - glycosylated cyclic pentamer which increases in concentration in a fixed and phased sequence during inflammation and / or active tissue infections. It is important in protection against development of autoimmunity. It acts by clearing cell debris autoantigens recognised by auto antibodies. The rise in CRP levels in DCM patients can be attributed to this factor. Rheumatoid factor (RF) was absent in all the sera of DCM
patients. RF are credible markers in inflammatory diseases of the joints. Hence their absence rules out the possibility of any such factor in DCM.

Autoantibodies found in patients with dilated cardiomyopathy (DCM) exhibited remarkable binding with the HPLC purified 150kD human C-protein as is evident from competition inhibition ELISA results. Nearly all the sera of patients with DCM showed inhibition in the antibody activity by 150 kD C-protein in the range of 64.7% to 82.1%. Furthermore, an appreciable number of DCM sera exhibited inhibition in their antibody activity by a very low magnitude of inhibitor concentration, which in turn, is further substantiative/or indicative for the remarkably high specificity of the DCM autoantibodies towards 150kD C-protein. However, the binding towards 150kD C-protein was of higher magnitude with the induced anti 150 kD C-protein antibodies in comparison to the binding with DCM autoantibodies. The binding results are suggestive for the major involvement of 150kD C-protein as autoantigen in autoimmune DCM/myocarditis.

It is to be pointed out that native DNA was found to be of low reactivity with all the sera of patients with DCM. But interestingly, mitochondrial DNA (mt DNA) on the other hand, exhibited appreciable binding with DCM autoantibodies, although the magnitude of binding was lower than with 150kD C-protein. Moreover, surprisingly, modification of mt DNA with ROS showed decrease in binding with DCM autoantibodies, whereas conversely, nDNA which was unreactive towards DCM autoantibodies, showed a binding of low magnitude when modified with ROS. The results are suggestive for the probable presence of conformational epitopes on mtDNA and to a
lower extent on ROS DNA which are somewhat similar to the ones present on 150 kD C-protein.

The highly immunogenic nature of native 150kD C-protein was evident from the competition results, where around 98 percent of the antibody activity was inhibited by the immunogen. However, when native 150kD C-protein was ROS modified, the antibody binding was lost to the extent of more than fifty percent. The results suggest for the damage or loss of significant number of immunodominant epitopes on 150kD C-protein upon ROS modification.

Although the induced anti-150kD C-protein antibodies were unreactive with native DNA, a low magnitude of binding was observed when nDNA was photoadducted with C-protein, whereas, surprisingly, ROS-DNA-C-Protein photoadduct exhibited a high degree of binding which was even higher than the binding observed with the immunogen i.e. 150kD C-protein. This could be attributed to the cumulative binding effect of immunogenic epitopes on C-protein as well as somewhat similar epitopes to the immunogen on the ROS modified DNA and hence when both ROS-DNA and C-protein were photoadducted, a higher binding was observed. The results suggest that probably apart from 150kD C-protein, the photoadducted ROS-DNA C-protein complex could also act as an alternate autoantigen for myocarditis/DCM. The above possibility could not be ruled out because of the fact that reactive oxygen species (ROS) are formed in vivo which modifies the nucleic acid macromolecule and that the possibility of photoconjugation of ROS-DNA with C-protein exists. Thus, our findings could possibly throw some light on the pathogenesis and etiology of autoimmune myocarditis/DCM.
The binding of induced anti-C-protein antibodies with histones, ROS-histone, poly-D-lysine, poly-L-glutamate and poly (lysine-glutamate) complex was of low magnitudes thereby suggesting for the presence of a minor sub-population of epitopes responding to the anti-C-protein antibodies. Similar binding results were also observed with DCM autoantibodies against C-protein, ROS C-protein ROS-DNA, ROS-DNA photoadducted to C-protein, ROS-histone, histones, poly-D-lysine, poly-D-glutamate and poly (L+G) complex respectively. The similarity in binding results further substantiates the above argument.

Apart from the above the systematic probe to see the correlation between myocarditis autoantigen (C-protein) and autoimmune SLE autoantibodies gave encouraging results.

Systemic lupus erythematosus (SLE) is a multisystem prototype autoimmune disease (Stollar, 1981) characterized by circulating autoantibodies that bind to cellular antigens of self and exogenous origin. Although the correlation of SLE with immune system is well documented, neither the origin of these autoantibodies nor the etiology of the disease is known. Recent studies point out that native DNA might actually be a cross reactive antigen whereas some other structures like modified nucleic acids (Alan et al., 1993; Ara and Ali; 1993; Ara and Ali, 1992), polypeptides (Arif et al., 1994) and phospholipids (Smeenk et al., 1987), etc. stimulate the antibody response. Moreover, the polyspecificity of autoantibodies with a variety of heteroantigens might be the result of immune response to substance (s) other than native DNA and interplay of some environmental factors.
In the present study attempts were also made to probe the possible involvement of 150 kD C-protein isolated from human heart which is known as potential autoantigen in myocarditis and DCM in humans, in autoimmune SLE. The protein is known to have similar domain as to those of IgG and has been implicated in the induction of autoantibodies in patients with SLE. The 150 kD C-protein, which is a component of thick filament of skeletal and cardiac muscles, plays an important role in the contractile activity. Assays of anti-DNA and anti-human heart antigens in the sera of patients with SLE and DCM respectively were carried out by employing direct binding ELISA whereas specificity determination was accomplished by competition ELISA. The antigen binding characteristics of anti-DNA autoantibodies towards native and modified nucleic acids were in accordance with earlier findings (Losman et al., 1993).

The most striking finding of the present study is the appreciable recognition of THHE as well as 150 kD C-protein (human heart) by SLE autoantibodies. As revealed by competition inhibition studies, the SLE autoantibodies expressing specificity towards dsDNA, ssDNA, DNA-lysine photoadduct and Br-DNA also showed almost similar recognition of human heart proteinic antigen(s). Interestingly, as evident from the binding results the human heart extract antigens reactive with the DCM autoantibodies showed strong recognition of SLE anti-DNA autoantibodies whereas on the contrary DNA was found to be less reactive with DCM autoantibodies. This is suggestive for the possible involvement of a common or somewhat similar trigger/epitope(s) for immune response in autoimmune myocarditis/DCM and autoimmune SLE, although such inferences could not lead to confirmity at such a preliminary stage of investigation. Moreover, the high binding
specificity of immunoaffinity purified anti-DNA antibodies to 150 kD C-protein from human heart is strongly suggestive for the presence of immunodominant epitopes on 150 kD C-protein for SLE anti-DNA antibody binding. The binding results are indicative for the presence of unique conformational epitopes on the 150 kD C-protein whose domains are somewhat similar to IgG. It is to be pointed out that recently nucleosome specific antibodies have been indicated in lupus prone mice. The monoclonal antibody was found to react with nucleosome core particle but not histone-histone and histone-DNA complexes. The core particles having ordered association of histones and DNA were found to express a unique conformational epitope(s). Only the pathogenic T-helper cells of lupus prone mice responded to nucleosomal antigens and their stimulation was specific (Chandra et al., 1993). Therefore, it appears that pathogenic anti-DNA autoantibodies are generated through some conformational epitope(s) of nucleic acids or proteins/polypeptides.

Thus, in view of the above argument, it appears from our data that 150 kD C-protein having domains similar to those of IgG and being involved in the induction of autoimmune myocarditis may provide some unique conformational epitopes which could possibly act as an alternate autoantigen or trigger in the production of anti-DNA autoantibodies in SLE. Our preliminary investigation is supportive for the above argument. The mechanism or the role of myocarditis inducing 150 kD human heart C-protein in the stimulation of immune system for the production of antibodies in SLE remains to be ascertained.
In conclusion, it would be inferred from this study that:

1. 150kD C-protein is having somewhat similar domains to human IgG as indicated by urea SDS-PAGE.
2. 150kD C-protein was thermodynamically more stable than human IgG although both of them had somewhat similar domains.
3. The C-protein is a potent immunogen inducing high titer polyclonal antibodies with major reactivity towards HPLC purified immunogen.
4. Nearly all the DCM sera tested exhibited elevated C-reactive protein (CRP) and uric acid levels and that all of them showed remarkable recognition towards electroeluted, followed by HPLC purified 150kD C-protein.
5. The kinetics of formation of DNA-C-protein photoadduct at various irradiation time obeyed the apparent first order reaction.
6. Out of the various adducts/photoadducts formed by crosslinking native DNA with C-protein, the one formed in the presence of OH (ROS -DNA-C-protein photoadduct) showed highest reactivity with DCM, SLE and immune IgG.
7. Nearly all the DCM sera tested, showed preferentially higher binding with native mitochondrial DNA than its ROS modified conformer.
8. The most striking finding is the appreciable recognition of THHE as well as 150 kD cardiac C-protein by immunoaffinity purified anti-DNA SLE autoantibodies.